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ATTEMPT TO CLONE MARROW CELLS IN SPLEENS OF LETHALLY IRRADIATED RHESUS MONKEYS

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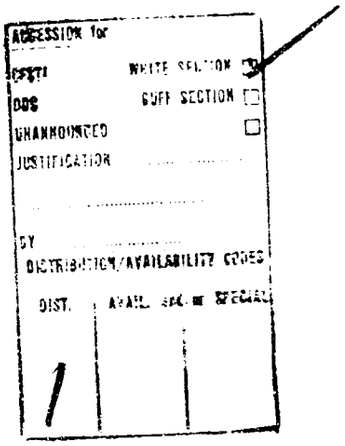

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FOREWORD

This research was conducted in the Radiobiology and Biosciences Divisions under tasks Nos. 775703 and 775311. The work was performed between 1966 and 1969 and the paper was received for publication on 18 August 1969.

The animals involved in this study were maintained in accordance with the "Guide for Laboratory Animal Facilities and Care" as published by the National Academy of Sciences–National Research Council.

Grateful acknowledgement is made to Master Sergeant Jesse Vasys for histologic assistance.

This report has been reviewed and is approved.

Jos M Quashnock

JOSEPH M. QUASHNOCK
Colonel, USAF, MC
Commander

ABSTRACT

An attempt was made to clone rhesus marrow cells within the spleens of lethally irradiated rhesus monkeys. Twelve animals received autologous transplants; 2 animals received allogeneic transplants; and 4 animals served as irradiation controls. Ten days following transplantation (or irradiation, in the case of the controls) the animals were sacrificed and their spleens removed. No macroscopic nodules were found in any of the spleens; microscopic examination of subserial sections revealed scattered erythrocytic clones in 3 of 14 animals that received transplants. Reticulocyte co[illegible] peripheral leukocytes, and cellularity of marrow spaces indicated that the transplanted marrow had proliferated in 13 of the 14 animals with transplants. Under our conditions, significant marrow cloning did not occur in spleens of lethally irradiated rhesus monkeys in a manner analogous to that described in similar studies with rodents.

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I. INTRODUCTION

The ability of murine marrow cells to form clones in the spleens of lethally irradiated mice as described by Till and McCulloch (5) has proved a useful method for investigating cellular and marrow kinetics. Recently this technic has been extended to the rat by Comas and Byrd (1). Attempts to clone rhesus marrow cells in the spleens of lethally irradiated mice have yielded essentially negative results in our laboratory. This may be the result of rejection of the xenogeneic marrow, nonspecific cytotoxic antibodies, or a fundamental difference between primate and rodent stem cells. Several species normally contain hematopoietic tissue within their spleens throughout life, but the rhesus monkey is not included in this group. One might speculate that attempts to clone marrow cells within primate spleens in a manner analogous to that used in rodents would be destined to fail for lack of a proper environment in the primate spleen or because of a difference in the cells responsible for repopulating the marrow spaces, or for both of these reasons. A successful method of cloning marrow in primate spleens would permit extension of some basic radiation biology technics to a higher animal. Attempts were, therefore, made to accomplish this.

II. METHODS

Eighteen rhesus monkeys, ranging in weight from 2.5 to 4.0 kg., were used as subjects in this study. The transplantation technic has been described in detail elsewhere (2), but may be briefly summarized as follows: Bilateral aspiration of femoral marrow was performed under sodium thiopental anesthesia. During and after aspiration the marrow was mixed with approximately 200 ml. of chilled, heparinized TC-199 medium (Difco) and stored in ice until centrifuged. Following centrifugation, the majority of the supernatant fluid was removed so that the final volume of the marrow mixture was 20 to 30 ml. This was stored at 2° to 4° C. until infused into a leg vein of the recipient animal.

After recovery from anesthesia, the animals received a midline air exposure of 1,000 R (830 rads) from a cobalt-60 source via anterior and posterior fields at a rate of 100 R per minute. Exposure doses were measured in air with a Victoreen high-energy chamber placed in a position corresponding to the midline of the irradiated animal. The calculated absorbed dose was based on determinations made in a Masonite monkey phantom.

The animals were divided into 4 groups: Group 1, a control group of 4 animals that received 25 ml. of TC-199 after irradiation; group 2, 6 animals that received autologous marrow 1 to 2 hours after irradiation; group 3 6 animals that received autologous marrow 24 hours after irradiation; and group 4, 2 animals that received allogeneic marrow transplants 1 to 2 hours after irradiation.

Penicillin (300,000 units) and 40 mg. of tetracycline were administered intramuscularly once a day from the second postirradiation day until sacrifice. Baseline peripheral blood samples were obtained immediately before marrow aspiration and additional samples were

obtained 7 and 10 days posttransplantation (or postirradiation, in the case of the control group). The 10-day sample was obtained at the time of sacrifice.

All animals were sacrificed by intravenous sodium pentobarbital. The spleens were immediately removed, sliced at 5-mm. intervals, and fixed in either buffered formalin or Bouin's solution. After fixation, sections (6μ thick) were cut from paraffin-embedded specimens. All blocks were serially sectioned, and every 14th and 15th section mounted on a glass slide and stained with either hematoxylin and eosin (H & E) or Giemsa. Similar preparations were made of sections from the sternum and the eighth thoracic vertebra

III. RESULTS

Since the aspirated marrow contained an unknown quantity of blood, the marrow cell counts have been expressed as the number of nucleated cells infused per kilogram. The number of nucleated cells infused, the reticulocyte and leukocyte counts, and a subjective estimate of the bone marrow cellularity are presented in table I. With the exception of

TABLE I

Peripheral blood data, preirradiation and postirradiation, number of nucleated cells infused, and marrow cellularity at sacrifice

Group	Animal number	Leukocytes ($\times 10^3$/mm.^{3})			Reticulocytes (%)			Number of cells infused per kg. ($\times 10^8$)	Average marrow cellularity (%)
		Baseline	Day 7	Day 10	Baseline	Day 7	Day 10		
1 — No bone marrow	1	17.2	0.20	0.45	0.3	0.0	0.0	—	Aplastic
	2	10.3	0.25	0.30	0.4	0.0	0.0	—	Aplastic
	3	13.2	0.30	Hemolyzed	0.8	0.0	Hemolyzed	—	Aplastic
	4	10.4	0.20	Dead	0.6	0.0	Dead	—	Aplastic
2 — Autologous marrow, 1 to 2 hr. postirradiation	5	8.8	0.30	0.90	0.1	0.0	0.3	2.9	30
	6	8.3	0.40	0.60	0.5	0.0	0.5	3.1	40
	7	7.5	0.30	0.90	0.2	0.0	0.7	3.6	55
	8*	7.8	0.20	0.65	0.5	0.0	0.1	1.8	5
	9	9.7	0.20	0.45	0.6	0.0	0.3	5.2	25
	10	11.3	0.15	0.35	0.3	0.0	0.5	5.4	20
3 — Autologous marrow, 24 hr. postirradiation	11†	8.2	0.25	1.00	0.6	0.0	0.5	4.1	20
	12‡	12.7	0.25	0.80	0.5	0.0	0.3	4.0	30
	13	11.8	0.25	1.40	0.3	0.0	0.7	4.7	35
	14†‡	20.0	0.45	1.00	0.2	0.0	0.4	6.7	20
	15	9.7	0.30	1.00	0.1	0.0	0.3	4.6	20
	16	11.1	0.40	0.80	0.4	0.0	1.0	4.6	25
4 — Allogeneic marrow, 1 to 2 hr. postirradiation	17	15.7	0.50	0.25	0.2	0.0	0.7	3.5	15
	18†‡	12.7	1.10	0.95	0.3	0.1	1.3	5.6	75

*Probably no marrow take.

†Microscopic splenic clones.

‡Grossly normal spleen.

animal 8, by the 10th day there was evidence of marrow proliferation in the transplanted groups as reflected by the rising leukocyte and reticulocyte counts. The low reticulocyte count of animal 8 on day 10 suggests that the transplanted marrow did not take in that animal. This animal received the lowest number of nucleated cells per kilogram and exhibited the least degree of marrow cellularity (table I). One of the 4 animals which did not receive marrow was dead on the morning of the 10th day; the 10-day blood sample of another was hemolyzed. In the other 2 control animals, the 10-day reticulocyte counts were zero, a finding which concurs with previously published data from our laboratory showing no evidence of recovery after 11 days in monkeys receiving 980 R (810 rads) under similar conditions (2).

On gross examination, the majority of the spleens appeared relatively small with grayish, contracted capsules. The spleens from 2 animals that received autologous marrow 24 hours post-irradiation and from 1 animal that received an allogeneic transplant were normal in size and had a brownish-red appearance. In no instance were nodules grossly visible beneath the capsule or on a cut surface in either the fresh or fixed state.

In the group not receiving marrow transplants, splenic sections were characterized histologically by lymphocytic depletion of the follicular areas. All that remained in these areas were reticulum cells and an occasional lymphocyte or plasma cell. Germinal centers were not present and mitotic figures were not seen in the follicular areas. The red pulp was not remarkable and sinusoids contained small to moderate numbers of mature erythrocytes. Scattered sinusoidal lining cells had hemosiderin granules within their cytoplasm. Bone marrow sections were characterized by nearly complete cellular aplasia with widely isolated, small clusters of immature erythrocytic cells together with normal components of endothelial, reticulum and plasma cells (fig. 1).

FIGURE 1

Marrow section from animal 4. Marrow spaces are essentially devoid of cells. Capillaries contain numerous erythrocytes. H & E × 40.

Eleven of the 12 animals that received autologous marrow had microscopic evidence of marrow takes as reflected by the cellularity of the marrow spaces (table I). Neither bone marrow nor splenic sections from animal 8 showed evidence of significant marrow or lymphocytic cell proliferation.

The following results apply to the remaining 11 animals receiving autologous marrow transplants. Widely scattered small, microscopic clones of immature erythrocytes were present in the spleens of animals 11 and 14 (fig. 2). The erythrocytic clones were small, consisting of less than 50 cells, most of which were at a rubricyte stage of development. Mitotic figures were visible within these clusters of immature erythrocytes. Three additional animals that received autologous marrow 24 hours postirradiation and one that received autologous marrow on the day of irradiation had widely scattered small clusters or rows of nucleated erythrocytes, 4 to 8 cells, in sinusoidal areas. The absence of mitotic figures made it questionable if these cells arose *in situ*. In 10 animals, scattered individual nucleated red cells, rubricyte types, were visible within the splenic sinusoidal lumens indicating they were free within the circulatory channels. Mitotic figures were occasionally observed in sinusoidal areas, but the cell types could not be identified with certainty. In none of the sections were clones of granulocytic cells seen although small to moderate numbers of band- and segmented neutrophils were present in sinusoidal lumens in 11 animals. Evidence of repopulation of the splenic follicular areas was present in the 11 animals as varying numbers of lymphocytes and mitotic figures were easily distinguishable. In 4 animals, repopulation had progressed to the extent that small germinal centers had reformed. Subjective appraisal of sternal marrow sections from the 11 animals revealed 20% to 60% of the marrow

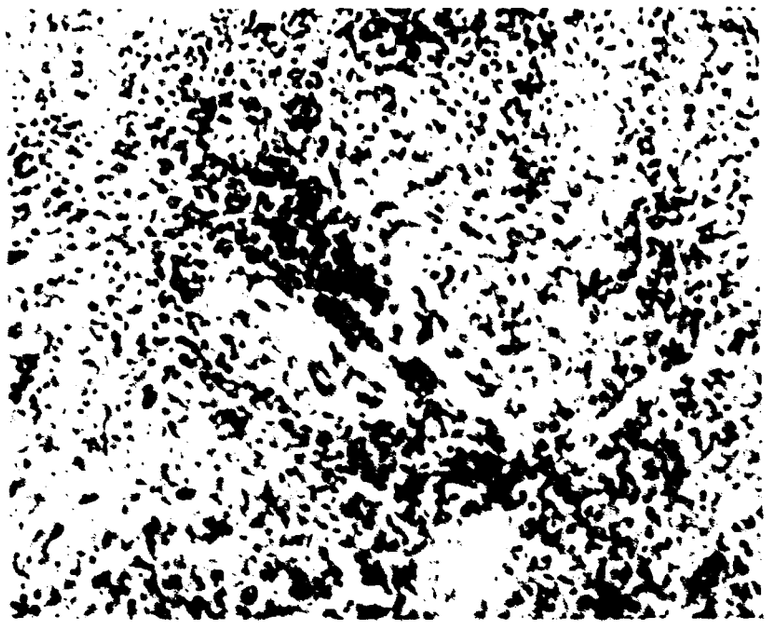

FIGURE 2

Splenic section from animal 14 illustrating a small irregularly shaped cluster of nucleated erythrocytes, 10 days after autologous marrow transplant. The dark-staining nucleated erythrocytes are located near a follicular artery. H & E × 215.

spaces to be occupied by cellular elements compared to 10% to 50% in vertebral sections (fig. 3). Cell types included those of the granulocytic series, erythrocytic series, and megakaryocytes.

One of the 2 animals (No. 18) which received allogeneic bone marrow had 3 clones of immature erythrocytic cells around splenic central arteries (fig. 4). These clones contained mitotic figures and one clone extended through sections taken over 100μ apart. Splenic follicles in each of these animals showed cellular repopulation with large blast-type cells of the lymphoreticular variety. Numerous mitotic figures were interspersed among these cells. The red pulp showed focal areas of hemorrhagic necrosis and marked proliferation of sinusoidal lining cells. Marrow sections from animal 18 showed normal cellularity as approximately 75% of the marrow spaces contained cellular elements; in the other animal that received allogeneic marrow, about 15% of the marrow spaces were occupied by cells.

IV. DISCUSSION

If adequate numbers of bone marrow cells are injected into lethally irradiated mice, it is possible to produce significant numbers of grossly visible splenic clones by 10 days after transplantation. Till and McCulloch (5) reported approximately one splenic colony per 10^4 nucleated cells injected in mice; similar figures have been reported by other authors. Comas and Byrd (1), using Fischer-334 rats, found 4.4 splenic colonies per 10^5 marrow cells injected. Senn et al. (4) have described the capacity of human bone marrow cells to form colonies on soft agar cultures and to produce an average of 34 colonies per 10^5 nucleated cells. In the present study the number of nucleated cells infused per kilogram was

FIGURE 3

Marrow section from animal 7. Marrow spaces show repopulation with cellular elements, a development which contrasts sharply with that of the irradiated, noninfused animal illustrated in figure 1. H & E × 40.

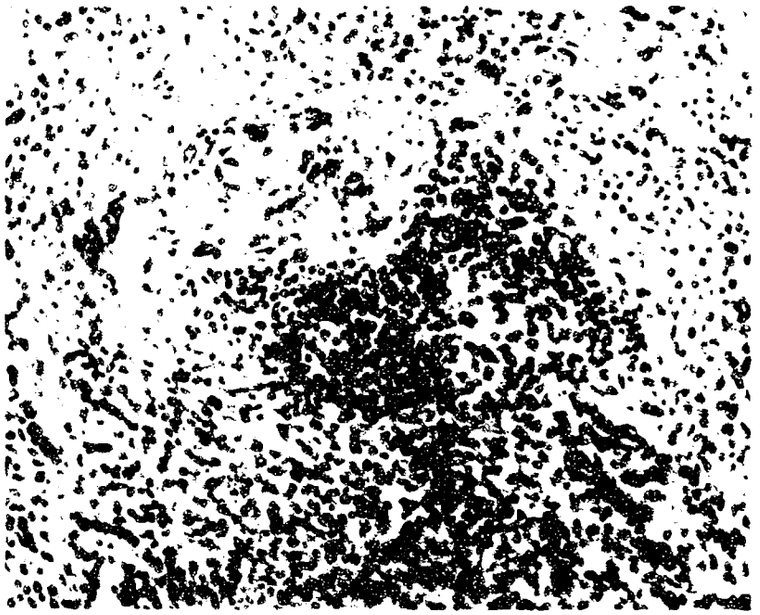

FIGURE 4

Small clone of nucleated erythrocytes in animal 18 that received allogeneic marrow 10 days previously. Dark-staining, nucleated erythrocytes are located in a follicular area. H & E × 215.

several orders of magnitude higher than that required to yield splenic colonies in rodents. This resulted in only scattered microscopic clones in the spleens of 3 out of 14 monkeys. Gross nodules were not found in any of the spleens. While the lack of a significant number of colonies at 10 days does not preclude their appearances at a later time, their presence would probably contribute little directly to the recovery of the animal. The evidence of lymphocytic repopulation in splenic follicular areas in 13 out of 14 marrow-infused animals demonstrated the ability of transplanted immature cells to seed and proliferate in one of their major sites of production. Nowell et al. (3) have demonstrated in mice that injected hematopoietic cells have a predilection to proliferate in sites in which they are normally found. The marrow and lymphocytic proliferation as described above in the monkey might also be considered an example of the "homing instinct" for these types of cells.

Thus, under the conditions described, rhesus marrow cells did not proliferate to a significant degree in the spleens of lethally irradiated rhesus monkeys. This observation, together with the evidence that transplanted marrow did proliferate elsewhere, suggests that significant splenic cloning may be limited to species in which the spleen normally hosts hematopoietic activity.

REFERENCES

1. Comas, F. V., and B. L. Byrd. Hemopoietic spleen colonies in the rat. Radiat. Res. 32:355-365 (1967).

2. Eltringham, J. R., D. R. Anderson and R. L. DeHart. Effect of azathioprine and amethopterin on secondary disease in the rhesus monkey. SAM-TR-67-94, Sept. 1967.

3. Nowell, P. C., et al. The distribution and *in situ* growth pattern of injected rat marrow in x-irradiated mice. J. Nat. Cancer Inst. 18:127-144 (1957).

4. Senn, J. S., E. A. McCulloch, and J. E. Till. Comparison of colony-forming ability of normal and leukemic human marrow in cell culture. Lancet 2:597-598 (1967).

5. Till, J. E., and E. A. McCulloch. A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. Radiat. Res. 14:213-222 (1961).

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13. ABSTRACT

An attempt was made to clone rhesus marrow cells within the spleens of lethally irradiated rhesus monkeys. Twelve animals received autologous transplants; 2 animals received allogeneic transplants; and 4 animals served as irradiation controls. Ten days following transplantation (or irradiation, in the case of the controls) the animals were sacrificed and their spleens removed. No macroscopic nodules were found in any of the spleens; microscopic examination of subserial sections revealed scattered erythrocytic clones in 3 of 14 animals that received transplants. Reticulocyte counts, peripheral leukocytes, and cellularity of marrow spaces indicated that the transplanted marrow had proliferated in 13 of the 14 animals with transplants. Under our conditions, significant marrow cloning did not occur in spleens of lethally irradiated rhesus monkeys in a manner analogous to that described in similar studies with rodents.

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